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PRIMARY SMALL-CELL LUNG CARCINOMAS AND THEIR METASTASES ARE CHARACTERIZED BY A RECURRENT PATTERN OF GENETIC ALTERATIONS

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Small-cell lung cancer (SCLC) represents a group of highly malignant tumors giving rise to early and widespread metastases. We used comparative genomic hybridization in autoptic tumor specimens from 10 patients to discover genetic alterations that are associated with tumor progression and potentially with the metastatic phenotype. Ten primary SCLC and 16 corresponding metastases were investigated with a maximum of 4 tumors per case. Prevalent changes observed in more than 60% of the primary tumors and their metastases included deletions on chromosomes 3p, 4q, 5q, 10q, 13q and 17p, and DNA over-representations on chromosomes 3q and 5p. The number of common alterations in the primary tumors and the related metastases outnumbered the differences, indicating a clonal relationship. Within the lesions of the same patient, differences were found between the primary tumor and the metastases as well as between metastases of distinct organ sites. However, no specific alteration was significantly associated with the metastatic phenotype. We suggest that the high malignancy of SCLC is defined by the above-mentioned pattern of aberrations. *Int. J. Cancer* 74:86–93.
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Lung cancer has become the leading cause of cancer-related death worldwide. Small-cell lung carcinomas (SCLC) comprise approximately one-fourth of lung-cancer cases. The majority of patients suffering from SCLC have metastases at the time of diagnosis. SCLC generally metastasize early to lymph nodes, adrenal glands, liver, lung, brain and bones. A combination of radiotherapy and chemotherapy for treatment of SCLC has improved the average survival time for patients with this disease. Nevertheless, the long-term prognosis is poor and the genetic basis for the aggressive biological behavior of SCLC is still poorly understood.

Several genetic alterations associated with SCLC have been reported. Changes in oncogenes involve genes of the *myc* and *ras* families (Brennan *et al.*, 1991; Takahashi *et al.*, 1989), as well as mutations of tumor-suppressor genes such as *p53* and *RB1* (Sameshima *et al.*, 1992; Kelley *et al.*, 1995) and the recently identified *FHIT* gene (Sozzi *et al.*, 1996). Furthermore, other deleted regions were identified at chromosomes 4q, 5q and 10q. Additional overrepresentations of chromosomes were observed at 3p, 5q and 19q (Ried *et al.*, 1994; Petersen *et al.*, 1997).

So far there are only a few indications as to which genetic alterations among the multitude of changes might be preferentially associated with the metastatic phenotype. Therefore, we used comparative genomic hybridization (CGH) to screen the genomes of 10 primary SCLC and 16 corresponding metastases. This method is based on competitive *in situ* hybridization of differently labeled DNAs (one from the tumor and another from the normal reference) to normal metaphase spreads. It allows a survey of DNA copy number changes (Kallioniemi *et al.*, 1992). The distinct advantage of CGH is the detection and mapping of DNA imbalances in the entire tumor genome compared to the investigation of single loci by other methods. Our analysis indicated common changes, *i.e.* alterations present in the primary tumor and in at least 1 metastasis, comprising deletions on chromosomes 3p, 4q, 5q, 10q, 13q and 17p, and over-representations on chromosomes 3q and 5p. Despite this recurrence, the patterns varied from one case to another, whereas the tumors of 1 patient showed a clonal relationship. Additional changes found in the metastases were 20 DNA over-representations and 17 deletions. Aberrant alterations of the primary tumors compared to the metastases comprised 7 DNA gains and 2 losses. The most prevalent changes were observed in primary tumors and metastases at a similar incidence. Hypothetically, they are responsible for the malignant phenotype of SCLC, including metastases formation.

MATERIAL AND METHODS

Tumor specimens

The tumor samples were collected at autopsy performed 8 to 36 hr after the patient's death. The clinical and histopathological data are summarized in Table I. Six patients were female and 4 were male. All specimens were frozen in liquid nitrogen and stored at −80°C until DNA extraction. Tumors were classified according to the TNM staging system.

DNA preparation

Control genomic DNA was extracted from blood leukocytes of a healthy donor. Tumor and normal DNA were prepared by proteinase K digestion and phenol/chloroform/isoamylalcohol extraction. Since SCLC shows a solid growth pattern, almost without intervening normal tissue stroma, no microdissection was necessary. The amount of tumor tissue, as assessed by cryostat section stained with H. and E., exceeded 90% in each case.

Chromosome preparations

Metaphase spreads for CGH were prepared from phytohemagglutinin-stimulated lymphocytes of healthy individuals (46, XX or 46, XY) by standard procedures. The slides with the metaphase spreads were stored at room temperature until use. Each batch of metaphases was tested by the hybridization of normal DNA as recommended (Kallioniemi *et al.*, 1994). In general, no pepsin or protease treatment was performed.

DNA labeling

Tumor DNA was labeled with biotin-16-dUTP (Boehringer Mannheim, Germany) by the standard nick translation reaction (Ried *et al.*, 1994). Control DNA was labeled accordingly by digoxigenin-11-deoxyuridine triphosphate incorporation (Boehringer Mannheim). The DNase I concentration in the labeling reaction was adjusted to yield an average fragment size varying between 200 and 500 bp.

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TABLE I – CLINICOPATHOLOGICAL DATA

Case	Diagnosis	Sex	Age	Stage	pM1[1]	Specimen analyzed[1]
1	SCLC	M	83	pT3 pN3 pM1	bo, he, li, lg, pl	pt, li
2	SCLC	F	57	pT3 pN2 pM1	ad, bo, li	pt, li
3	SCLC	M	75	pT4 pN3 pM1	ad, bo, he, ki, li, lg	pt, li, ad
4	SCLC	F	63	pT3 pN3 pM1	ad, br, ki, ov, pa, th	pt, ad, ki
5	SCLC	F	66	pT3 pN3 pM1	ad, ki, li, pa, th	pt, ad
6	SCLC	F	46	pT4 pN3 pM1	ad, bo	pt, ly
7	SCLC	F	79	pT4 pN3 pM1	ad, lg	pt, lg, ad
8	SCLC	F	58	pT4 pN3 pM1	ad, br, he, lg, li, pa, sk, th, mp	pt, lg, li, mp
9	SCLC	M	80	pT4 pN3 pM1	ad, bo, li, pr	pt, li, ad
10	SCLC	M	50	pT4 pN1 pM1	br, he, li	pt, li

[1]*Abbreviations:* ad, adrenal gland; br, brain; bo, bone; he, heart; ki, kidney; li, liver; lg, lung; ly, lymph node; ov, ovary; pa, pancreas; pl, pleura; pr, prostate gland; pt, primary tumor; sp, spleen; sk, skin; th, thyroid gland; mp, minor pelvis; SCLC, small-cell lung carcinoma.

Hybridization

CGH analysis was carried out as described (Petersen *et al.*, 1997) with minor modifications. Metaphase spreads were denatured in 70% formamide and 2 × SSC (0.3 M NaCl, 0.03 sodium citrate) for 90 sec at 80°C, dehydrated in a sequence of 70%, 90% and 100% ethanol and air-dried.

Next, 1 μg each of biotinylated tumor and digoxigenin-labeled control DNA, together with 30 μg of human Cot-1 DNA (Gibco-BRL, MD), were precipitated in ethanol and dissolved in 15 μl of 33% formamide, 13.3% dextran sulfate, 2.7 × SSC. In our experience, this hybridization mix yielded better results than the standard one containing 50% formamide, 10% dextran sulfate, 2 × SSC. The probe mixture was denatured for 5 min at 77°C and allowed to pre-anneal at 37°C for 1 hr. Afterwards, 12 μl of this mixture were hybridized to the metaphase chromosomes for 2–3 days at 37°C.

Post-hybridization steps comprised the detection of the biotinylated DNA by avidin conjugated to fluorescein isothiocyanate (FITC, Vector Burlingame, CA) and digoxigenin-labeled DNA by tetraethylrhodamine isothiocyanate (TRITC, Boehringer Mannheim), respectively. Chromosomes were counterstained with 4,6-diamino-2-phenylindole (DAPI) and embedded in DABCO (Sigma, St Louis, MO) to reduce photobleaching.

Digital image analysis

Image acquisition was performed using an epifluorescence microscope (Zeiss Axiophot, Oberkochen, Germany) and a cooled CCD camera (Photometrics, Tucson, AZ) connected to a Macintosh Quadra 950. Digital image processing was facilitated by the software package KARYOTYP (IBSB, Berlin, Germany) which runs under Windows 95 or Windows 3.1 and was extended for CGH image analysis. It is based on the digital image analysis software AMBA which was developed in our laboratory.

Briefly, the digital image analysis comprised the following steps: (i) definition of the image objects (chromosomes) by segmentation of the inverted DAPI image; (ii) loading of the FITC and TRITC image under the DAPI segmentation mask; (iii) correction of the optical shift of the FITC and TRITC image; (iv) calculation of the RATIO (FITC/TRITC) image; (v) separation and karyotyping of the chromosomes (the DAPI, FITC, TRITC and RATIO chromosomes can be displayed during the karyotyping process by means of a "compare" function); and (vi) calculation of the mean ratio chromosomes (CGH sum-karyogram) and the mean ratio profiles by averaging at least 5 metaphases/karyograms.

In the CGH sum-karyogram (Fig. 1) a particular mean ratio chromosome is calculated from all chromosomes of the same class that are present in the individual karyograms. The DNA changes are represented by the 3 pseudocolors of the mean ratio chromosomes and the ratio profile at the right side of the chromosome ideogram. An over-representation of the tumor DNA compared to normal DNA is indicated by a green color of the chromosomes, whereas red stands for under-representations and blue represents an equilibrium between the tumor and normal genome. The ratio profile shows the result as a 2-dimensional curve. If the profile was clearly to the left of the monosomy threshold (monosomy in 50% of the tumor cells in an otherwise diploid tumor, *i.e.*, fluorescence ratio of 0.75), the change was scored as a deletion. Over-representations were assessed accordingly in relation to the trisomy threshold (fluorescence ratio 1.25).

In an extension of this concept, the CGH super-karyogram is calculated by averaging the chromosomes of the CGH sum-karyograms from different tumors that belong to a tumor subgroup, *e.g.* all metastases (Fig. 3).

Detailed information about the CGH preparation and the digital image analysis are available on our website http://amba.rz.charite.hu-berlin.de/cgh.

RESULTS

The clinicopathological data (Table I) indicated an advanced tumor stage in every patient with generally widespread metastases. Dissection of the pM1 stage showed that adrenal-gland metastases were most prevalent and occurred in 8 cases, followed by liver metastases in 7 cases. In total, we investigated 10 primary tumors and 16 metastases including 6 liver metastases and 5 adrenal gland metastases.

Figure 1 depicts the CGH sum-karyogram of the tumors of case 7. A pattern common to the primary tumor and the 2 metastases was observed on chromosomes 3, 4, 5, 10, 15, 16, 17 and 22. Differences were found on 2p and 13q. For chromosome 2p, the primary tumor showed an under-representation on the short arms that was not present in the metastases, whereas the lung metastasis retained the telomeric part of chromosome 13q which was lost in the primary tumor and the adrenal-gland metastasis. Fluorescence images of 10 metaphases were analyzed for each tumor.

Common changes

As exemplified by the tumors of case 7 (Fig. 1), a principal feature was that, in all cases examined, the number of common changes detected in different tumor sites of the same case outnumbered the differences. We performed a similar comparison of the CGH sum-karyograms of the other 9 cases and defined common alterations that are summarized in Figure 2. Common changes were defined as alterations present in the primary tumor and at least 1 metastasis. The prevalent deletions occurred on chromosomes 3p, 4q, 5q, 10q, 13q, 17p and the overrepresentations on chromosomes 3q and 5p. These aberrations were also indicated by the CGH super-karyogram of the 16 metastases (Fig. 3). In addition the latter one showed a DNA gain on chromosome 20q.

The tumors of one patient carried a mean of 12.9 common alterations (ranging between 5 and 21). The number of additional changes varied between 1 and 12 with a mean of 4.7.

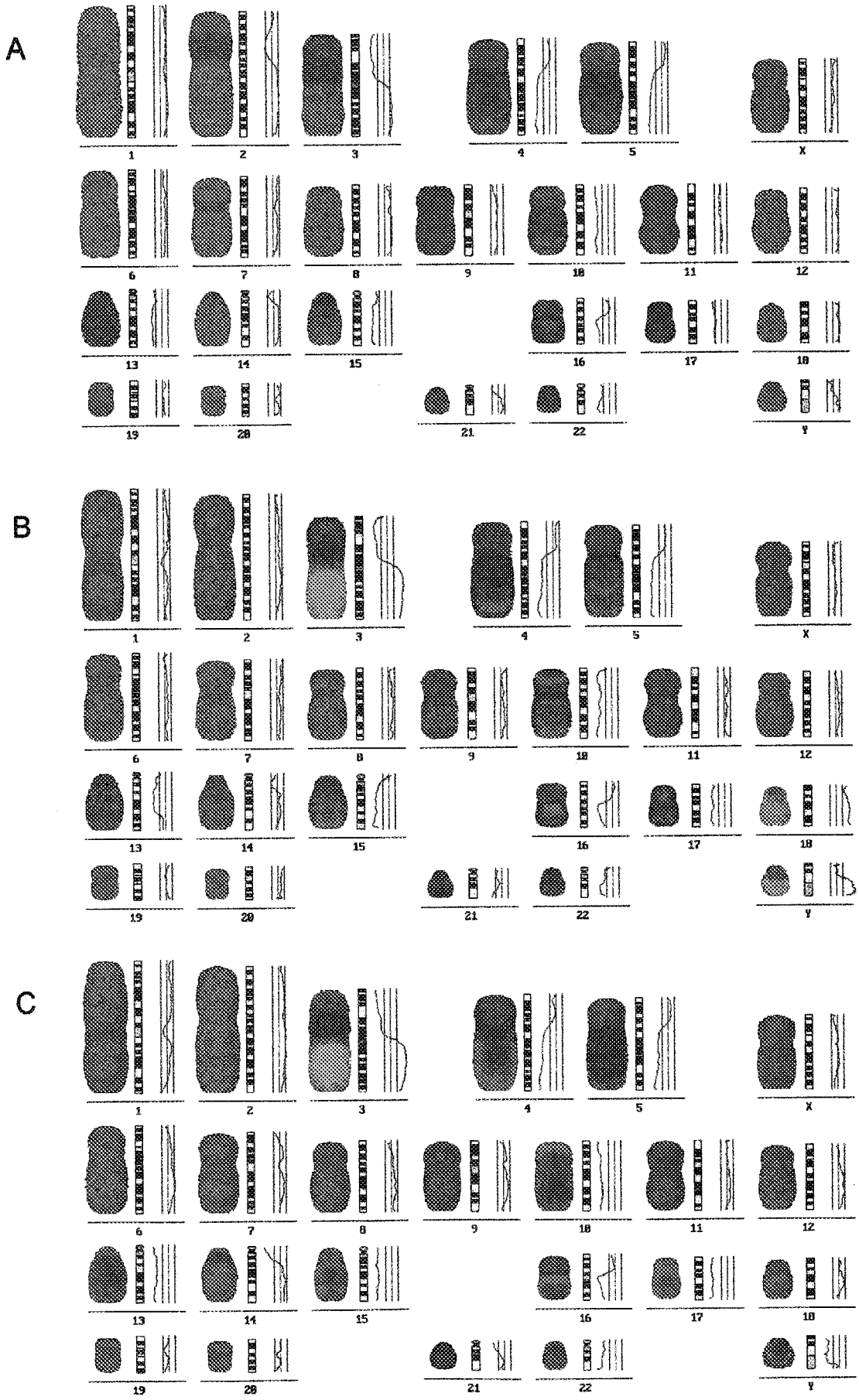
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FIGURE 1

TABLE II – GENETIC DIFFERENCES BETWEEN THE PRIMARY TUMORS AND THEIR METASTASES

Case	DNA gains[1]	DNA losses[1]
1	20q11.1-qter (li)	22q12-qter (li)
2		2p14–16 (li)
3	19q12–13.3 (pt), 21q11.1-qter (pt)	13q13-qter (li, ad), 22q11.2-qter (ad)
4	11q21-qter (ki), 13q33-qter (ad), 14q11.1-qter (ad)	7q11.2 (pt)
5	12q24.1–24.2 (ad)	
6	1p32-pter (bo), 3q (pt), 11q13 (bo), 16q22-qter (bo), 17p (bo), 22q (bo)	1p13–31 (bo), 3q21–23 (bo), 4 (bo)
7		2p13–16 (pt)
8	1p32–33 (li), 11p11.1–14 (pt), 13q12–13 (lg, mp), 19 (lg)	1p11–31 (li), 1p34.1-pter (li), 1q11-qter (li), 11q11-qter (lg), 13q14-qter (lg, mp), 16 (lg)
9	4p11–15.1 (pt), 7 (li, ad), 16 (ad), 20 (ad)	
10	6p21.3–22 (li), 6q11–22 (li), 4q11–24 (pt), 18q12-qter (pt)	4q11–24 (li), 5q14–23 (li)

[1]For abbreviations used, see footnote to Table I.

Differences

We observed differences between the primary tumor and the metastases as well as between different metastases of one patient (listed in Table II). Thirty-seven genetic changes were found in the metastases that did not occur in the primary tumors, *i.e.* 20 DNA over-representations and 17 deletions. In one instance, a metastasis retained genetic material that was lost in the primary tumor and in the other metastasis (see case 7, Fig. 1). Nine alterations (7 DNA gains and 2 deletions) were detectable in the primary tumors but not in the metastases (see Discussion). DNA gains of metastases that differed from the corresponding primary tumor were found twice on chromosomes 1p32-33, 7, 13q12-13, 16q22-qter, 19q12-13.3, 20q. DNA losses were observed 4 times for chromosome 13q13-qter and twice for chromosomes 1p13-31, 2p14-16, 4q11-24, 22q12-qter. No single aberration was significantly associated with metastatic phenotype or location of the metastases.

DISCUSSION

In the course of carcinogenesis, cells experience several genetic alterations that are associated with the transition from a preneoplastic lesion to an invasive tumor and finally to the metastatic state. Metastases are derived from single tumor cells that have invaded the blood vessels and grow in an organ away from the primary tumor. The primarily monoclonal nature of metastases is usually reflected by their morphological features, as they tend to form spherical tumor masses in parenchymal organs, *e.g.* the liver. We studied primary SCLCs and corresponding metastases in an attempt to identify genetic changes associated with the metastatic phenotype and to use this as a model for describing the genetic instability of this highly malignant tumor.

DNA over-representations

DNA amplification is generally considered to be a characteristic feature of tumor progression. DNA gains on chromosomes 3q and 5p were the most frequent finding. Candidate oncogenes on chromosome 3q are the CBLB gene, the ECT2 gene at 3q26, the ETV5 (ets variant gene 5) gene at 3q27-29, the EVI1 gene at 3q26, the RYK gene at 3q22 and the THPO gene at 3q26.33-27. They encode putative protein kinases, kinase receptors or transcription factors (details available in the GDB, internet website http://gdbwww.gdb.org). Some of these genes have been implicated in hematological malignancies; however there are no publications reporting alterations in lung carcinomas. To our knowledge, no candidate oncogenes have been identified on chromosome 5p.

Chromosome 20q showed an over-representation at 20q13 in 4 cases. This gain was found in the primary tumors as well as in the metastatic lesions. This alteration has been primarily observed in breast carcinomas (Kallioniemi *et al.*, 1994). The fact that it is also amplified in SCLC suggests that it harbors a gene that is generally implicated in tumor progression.

In case 8, in which the primary tumor and its 3 metastases were analyzed, all tumors showed an amplification at 2p22-24 which harbors the N-*myc* oncogene. In the liver metastasis, an additional amplification at 1p32-33 including the L-*myc* gene locus was present that could be detected neither in the primary tumor nor in one of the other 2 metastases. Similarly, an additional over-representation of 1p32-pter occurred in the bone metastasis of case 6. This corresponds to the fact that *myc* amplifications have been associated with advanced tumor stages (Takahashi *et al.*, 1989; Brennan *et al.*, 1991). Amplifications of the respective chromosomal region were particularly prevalent in SCLC cell lines (Levin *et al.*, 1994). However, we did not find recurrent over-representations of the *myc* gene regions or other chromosomal sites in the metastases. Instead, deletions were generally more prevalent. Therefore we hypothesize that predisposing conditions for the metastatic phenotype might be associated with deletions. Additional candidate genes regarding DNA over-representations have been discussed previously (Ried *et al.*, 1994; Petersen *et al.*, 1997).

DNA losses

The chromosomal regions which we found to be most often lost in SCLC were located on chromosome arms 3p, 4q, 5q, 10q, 13q and 17p.

Cytogenetic and molecular genetic analyses have indicated distinct subregions on chromosome 3p that showed deletions, *i.e.*, 3p14-23 and 3p25 (Whang-Peng *et al.*, 1982; Graziano *et al.*, 1991). In our study, each of 26 primary and metastatic SCLCs showed deletions of the entire short arm that were generally accompanied by an over-representation of the entire long arm, suggesting the formation of an isochromosome 3q. Isochromosomes are the cytogenetic correlate of low-level amplification of many genes which might induce a growth advantage important for tumor initiation. Deletions on chromosome 3p are considered to be an early event in the development of a varicty of tumors. Although isochromosomes 3q have been described in other tumors, *e.g.* head-and-neck squamous-cell carcinomas (Speicher *et al.*, 1995), these tumors more frequently carry interstitial deletions on 3p and amplifications of a telomeric 3q subregion. The prevalence of the i(3q) CGH equivalent in small-cell lung cancer (Ried *et al.*, 1994; Levin *et al.*, 1994; Petersen *et al.*, 1997) and particularly in

FIGURE 1 – CGH sum-karyogram of the tumors of case 7: *(a)* primary tumor, *(b)* lung metastasis, *(c)* adrenal-gland metastasis. Deletions are depicted in red, amplifications in green and equilibrium between the tumor and normal DNA in blue. Common changes that were present in all tumors are deletions on chromosomes 3p, 4q, 5q, 10, 15q, 16q, 17, 22 and over-representation of 3q. Differences between the tumors were observed on chromosomes 2p and 13q. For chromosome 2p, the primary tumor showed an under-representation on the short arms that was not present in the metastases, whereas the lung metastasis retained the telomeric part of chromosome 13q which was lost in the primary tumor and the adrenal gland metastasis.

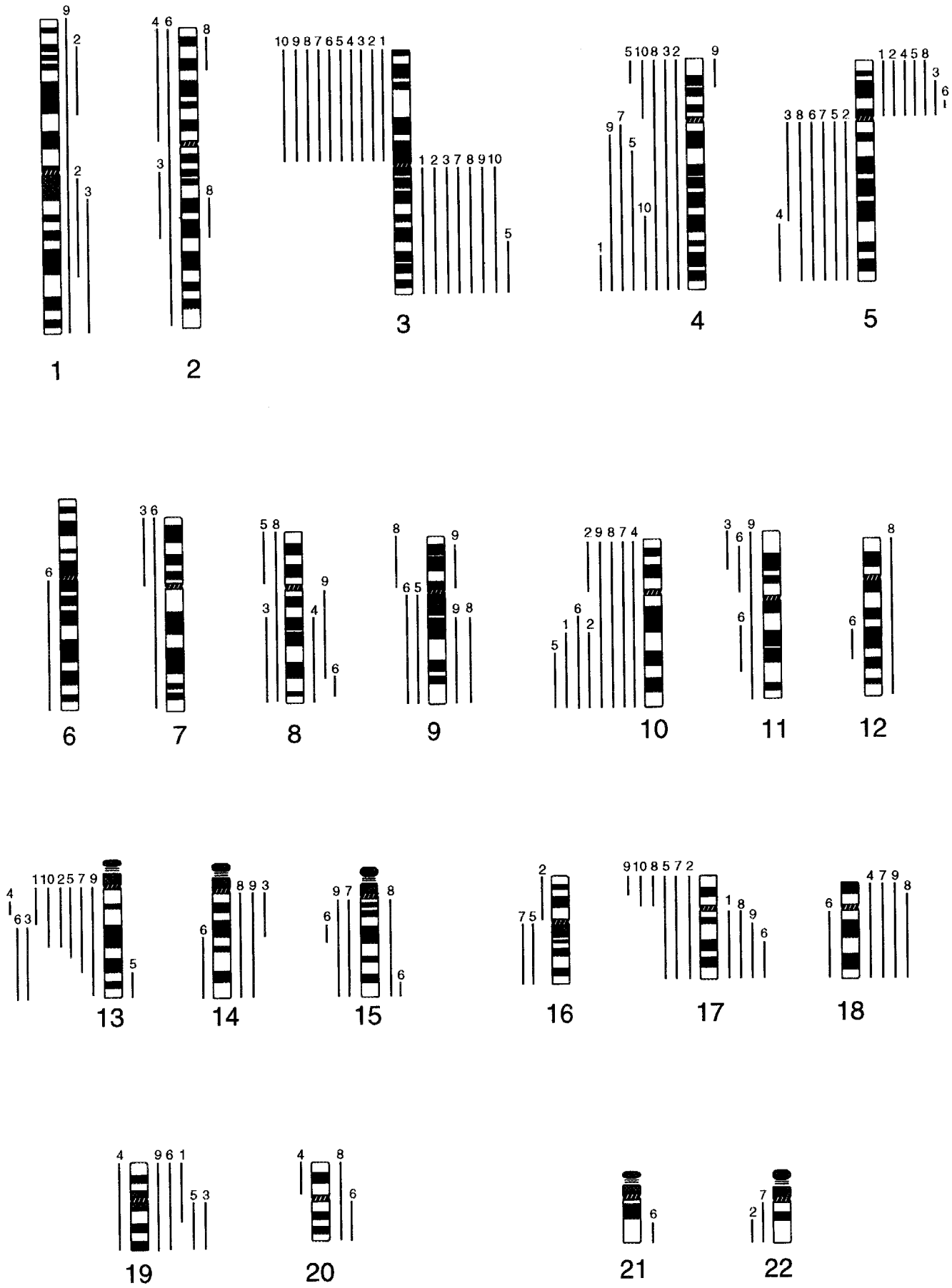

FIGURE 2 – Summary of common changes, *i.e.* alterations present in the primary tumor and at least one metastasis of each case (n = 10). Lines on the left of the chromosome ideogram represent a DNA loss, lines on the right DNA gains. The number at the top of each line corresponds to the case numbers of Table I. The prevalent changes, *i.e.* present in more than 60% of the cases, were deletions on chromosomes 3p, 4, 5q, 10, 13q and 17p and DNA over-representations on chromosomes 3q and 5p. A basic alteration was the DNA loss of the entire chromosome 3p which was observed in all cases.

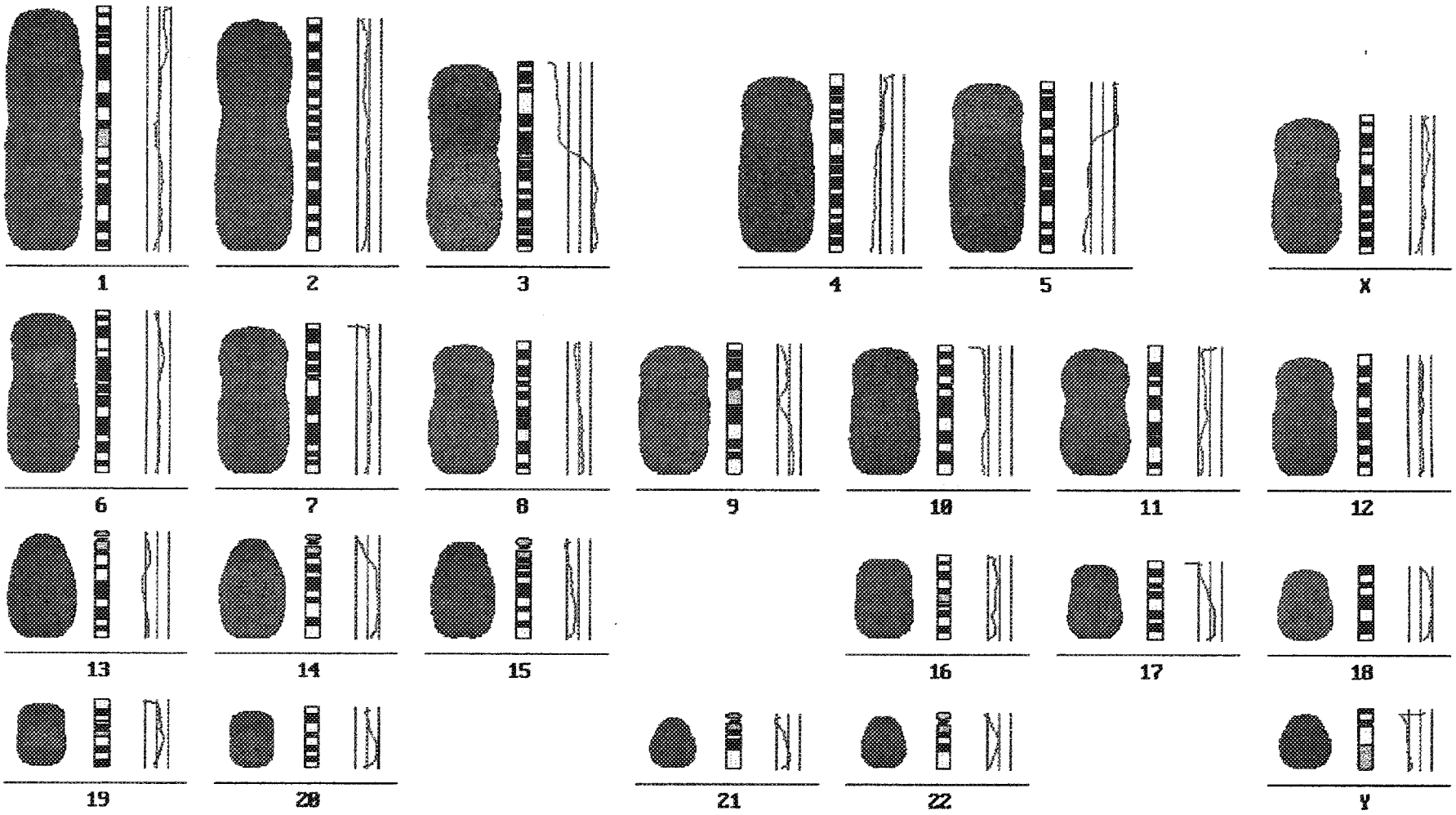

FIGURE 3 – The CGH super-karyogram of all 16 metastases of the 10 cases clearly indicates the prevalent changes on chromosomes 3, 4, 5, 10, 13 and 17. In addition, chromosome 20q shows an over-representation, suggesting that this chromosomal region harbors a gene implicated in tumor progression.

metastasizing SCLC might indicate that this change is characteristic of tumor progression.

The loss of 5q was observed in 70% of cases. In the majority of tumors, the entire chromosomal arm was lost. In one tumor we found a minimal centromeric region of deletion at 5q14-23 (liver metastasis of case 10). The region 5q13-21 has been reported to be preferentially deleted in SCLC (Miura *et al.,* 1992; Ried *et al.,* 1994) and candidate tumor-suppressor genes have been mapped to this region, *e.g.* the *APC* and *MCC* genes. The role of these genes in lung carcinogenesis, however, has not yet been elucidated. In addition to the loss of 5q, a simultaneous gain of 5p with the putative formation of i(5p) occurred in 3 cases. Additionally, 2 cases with a 5q loss showed partial over-representations of 5p. A similar pattern was observed for chromosome 17 in which deletions on one chromosomal arm were accompanied by over-representations of the other chromosomal arm. This is consistent with cytogenetic studies indicating that these chromosomes in particular exhibit chromosomal breakpoints (Miura *et al.,* 1992) and might indicate that they are prone to intrachromosomal rearrangements.

A single report described 4q deletions in 6 of 16 (37.5%) SCLC cell lines (Sekido *et al.,* 1993). We observed deletions in a centromeric region at 4q11-23 and a consensus telomeric region at chromosomal band 4q32. To our knowledge, no tumor-suppressor gene has yet been identified on this chromosome.

Deletions on chromosomes 13q and 17p generally included the loci of the tumor-suppressor genes *RB1* and *p53*. The high incidence of DNA loss in our series corresponds well to the fact that these 2 genes are frequently altered in SCLC (Kelley *et al.,* 1995; Sameshima *et al.,* 1992). In particular, *p53* mutations have been investigated in primary and metastatic tumors (Reichel *et al.,* 1994). The authors found that the metastases usually exhibited the same *p53* status as the primary tumors and concluded that the *p53* mutation generally precedes the development of the metastatic phenotype. In some cases, however, the mutation was found exclusively in the metastases or in the primary tumor. The data indicate that *p53* mutations *per se* are not responsible for metastasis formation. Deletions at the *RB1* locus are associated with tumor progression and poor survival in other tumor types. The recent finding that the functional RB protein represses gene transcription (White *et al.,* 1996) may indicate that the mutated gene could be permissive for aberrant gene expression associated with the malignant phenotype of tumor cells.

The loss of genetic material on chromosome 10q in SCLC has been previously observed (Ried *et al.,* 1994; Levin *et al.,* 1994), however at a lower incidence. Deletions on chromosome 10q have been described in other tumor types, *e.g.* gliomas, malignant meningiomas, endometrial cancer, melanomas and prostate cancer. In the present study it was the third most frequent deletion, together with losses on chromosomes 4q and 5q. We identified 10q23-qter as the shortest overlapping region. A candidate gene is the *MXI1* gene which is located at 10q24-25. It encodes a protein that negatively regulates the *myc* oncogene (Schreiber-Agus *et al.,* 1995). This is intriguing, since amplification and overexpression of *myc* oncogenes are frequent findings in SCLC (Brennan *et al.,* 1991) particularly in advanced tumor stages (Takahashi *et al.,* 1989). The *MXI1* gene has been suggested to act as a tumor-suppressor gene in prostate cancer (Eagle *et al.,* 1995) and it constitutes an attractive candidate for SCLC.

The fact that we failed to identify any DNA change that was significantly associated with the metastatic phenotype is probably due to the limited number of cases analyzed. Alternative mechanisms at the transcriptional and post-transcriptional levels have been reported to be involved in metastasis formation (Gunthert *et al.,* 1991).

Clonal relationship

The additional changes found in the metastases can be explained by the accumulation of genetic aberrations in the course of tumor progression. However, we also observed alterations in the primary tumor that were not detectable in the metastases (see Table II). The most likely explanation is that the primary tumor progressed genetically after the metastatic dissemination, *i.e.,* the predominant clone of the primary tumor no longer represents the metastatic clones that we investigated. Another explanation is sampling error, *i.e.* the tissue specimen taken from the mass of the primary tumor does not include the clones of the metastases.

As a main feature of our analysis, the number of common changes outnumbered the differences in any case, indicating the clonal relationship between the primary tumor and the metastases. This is in contrast to previous studies comparing primary tumors and metastases by using a single genetic marker, *e.g. p53* mutations (Reichel *et al.,* 1994) which failed to prove the clonality in every case. Thus, detection of a pattern of alterations is generally more suitable for genetic comparison of different tumors within a patient. In particular, the differentiation between a metastasis and a second primary tumor (Bedi *et al.,* 1996) might have profound clinical implications, because of the distinct therapeutic regimes. Since the majority of carcinomas are characterized by *a priori* unknown chromosomal imbalances, CGH is one method of choice to determine the clonal relationship between different tumors of a patient.

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